VICH DISCUSSION DOCUMENT

Prepared by: Gerald G. Christianson
Center for Veterinary Biologics
Ames, Iowa 50010

VICH Biological Quality Monitoring WG

Mycoplasma Guideline, March 2001 draft

Rewritten after Comments received on August 2000 draft.

1. INTRODUCTION

1.1. Objective of the guideline

It is important that biological products for veterinary use are free of contamination with Mycoplasma to help assure consistency of production and final product safety. Mycoplasma contaminants may be introduced into viral products in the master seed viruses (MSV), the master cell stocks (MCS), starting materials of animal origin, and in processing of biological materials during passage and product assembly. Therefore it is necessary to demonstrate through testing that Mycoplasma are not present, within the limits of the test, in the final product, working stocks and harvests, and starting materials such as the MSV, MCS**,** and ingredients of animal origin. This guideline establishes sampling and test procedures to assure the absence of Mycoplasma contamination. It will provide a unified standard to facilitate the mutual acceptance of test data by the relevant regulatory authorities.

1.2. Background

The present methods for testing for Mycoplasma contamination are described in the Japanese "Minimum requirements of biological products for animal use", the European Pharmacopoeia (Supplement 1998, 2.6.7), and the United States Code of Federal Regulations, Title 9, 113.28. These requirements are all similar in that they require testing for Mycoplasma contamination using a broth and agar technique. The requirements do however differ in the specifics of these broth and agar tests as well as other alternative test methods that are required or approved for use in detecting Mycoplasma contamination.

1.3. Scope of guideline

This guideline describes the manner in which cell culture and chicken embryonated egg origin biological products for veterinary use shall be tested to assure the absence of Mycoplasma contamination. These tests shall be applied as described to finished live products, and Master seed, Master cell, working seeds, working cells, harvests, and ingredients of animal origin for all cell culture and chicken embryonated egg origin products.

1.4. General principles

Mycoplasma contamination in master stocks, working stocks, ingredients of animal origin, harvests, and final live product will be tested by expansion in broth culture and

detection by colony formation on nutrient agar plates. Mycoplasma contamination in master and working seed and cell stocks will also be tested by expansion in cell culture and characteristic fluorescent staining of DNA, a technique capable of detecting non-cultivatable strains. (**TO BE DETERMINED 7**)

2. GUIDELINE FOR TESTING FOR MYCOPLASMA CONTAMINATION

2.1. Samples

(TO BE DETERMINED) Tests for Mycoplasma contamination shall be done on final container samples of each serial, lot, or batch of cell culture or chicken embryonated egg origin live vaccine. Pooled or single batch bulk harvest stages must be tested instead of the final product for killed vaccine and may be tested instead of final product for live vaccines. Each lot of master seed, primary and master cell stock, working seed, and ingredients of animal origin shall be tested for Mycoplasma contamination.

2.2. General test procedures for detecting Mycoplasma contamination

The culture method using broth and agar is the fundamental method of Mycoplasma detection. A solid and liquid media culture method shall be used to test final batches of vaccine and ingredients of animal origin. Master seed, master cell, and working seed and cell lots shall be tested using both a solid and liquid media culture method and an indicator cell culture method with DNA stain. Should either method result in a positive test for mycoplasma the sample is considered positive and is unsuitable for use.

2.3. Culture method

2.3.1. Laboratory validation for the culture method

The culture method should be carried out in a sufficient number of both solid and liquid media to insure the growth of approximately 100 CFU (70-130CFU) of the following 5 strains of mycoplasmas.

Acholeplasma laidlawii	Passage 1-15
Mycoplasma hyorhinis	Passage 1-15
Mycoplasma orale	Passage 1-15
Mycoplasma synoviae	Passage 1-15
Mycoplasma fermentans	Passage 1-15

The Master Reference strains of these organisms will be distributed to government and biologics industry laboratories which wish to be validated as capable to detect Mycoplasma contamination (**Appendix 3.2**). Those laboratories testing only mammalian products with no significant risk of exposure to avian origin ingredients will be exempt from testing *M. synoviae* as a reference organism. Those laboratories testing only avian products with no significant risk of exposure to mammalian origin ingredients will be exempt from testing *M. hyorhinis* as a control organism. Those laboratories testing products and ingredients that are free of antibiotics and preservatives will be exempt from testing *M. orale* and *A. laidlawii* as control organisms. Each laboratory must prepare Reference Strain Working Preparations from the master references

received at the time of their laboratory validation. These Working Preparations shall be used to validate each production lot of broth and agar. Whenever a laboratory deems it necessary to change a medium's composition or method of preparation, they must request new vials of Master Reference in order to revalidate their laboratory. At least one working reference strain must be used as a control with each test assay or test day. Each master reference will have its passage (one transfer followed by the normally used incubation period for the organism concerned) level marked on the vial and the passage level of each working reference should not exceed 15 passages with the master references passages included.

Required Reference Organisms by: product type; test method, and presence of antibiotics

Product type Test Method Antibiotics	*A. laidlawii*	*M. orale*	*M. hyorhinis*	*M. synoviae*	*M. fermentans*
Avian product Culture Method No-Antibiotics				X	X
Avian product Culture Method Yes-Antibiotics	X	X		X	X
Mammalian Product Culture Method No-Antibiotics			X		X
Mammalian Product Culture Method Yes-Antibiotics	X	X	X		X
Indicator Cell Method No-Antibiotics		X	X		
Indicator Cell Method Yes-Antibiotics	X	X	X		

2.3.2. Incubation conditions

Incubate the broth culture medium or media in air or microaerophilic (nitrogen containing 5-10% CO_2) conditions. Incubate all agar plates under microaerophilic conditions. For the solid medium or media, maintain an atmosphere of adequate humidity to prevent desiccation of the agar surface.

2.3.3. Nutritive properties of a new batch of medium

Each new lot (batch) of medium must be tested for the nutritive properties of working references specified above in **Section 2.3.1**. Each testing laboratory must

determine the inoculum for each of their frozen working references that will contain approximately 100 CFU (70-130 CFU). Inoculate the solid medium with approximately 100 CFU (70-130 CFU) per 60 mm plate and per 100 ml container of broth medium. Use at least one agar plate and broth container for each working reference. Incubate the agar and broth media and make subcultures from the broth onto agar at the specified intervals. The agar medium batch complies with the test for nutritive properties if approximately 100 CFU (70-130 CFU) are achieved for all the working references specified. The broth complies if Mycoplasma growth on those agar plates subcultured from the broth is achieved for each working reference specified. (**TO BE DETERMINED 8**) For each test session in which the validated batch of medium is used at least one of the specified working references must be used. Media formulations found effective are included in **Appendix 3.1** of this guideline.

2.3.4. Inhibitory substances

Carry out the test for nutritive properties in the presence and absence of the product to be examined at the time of prelicense and whenever the formulation of the product changes. If growth of the working references is (**TO BE DETERMINED 5**) absent on the agar plates subcultured from the broth containing product, and are present on agar plates sub-cultured from the broths containing no product, then the product contains inhibitory substances which must be neutralized or their effect otherwise countered, e.g., this may be achieved by dilution in a larger volume of medium, before the test for mycoplasma contamination is carried out. The effectiveness of the neutralization or other process is checked by repeating the test for inhibitory substances after neutralization. If the inhibitory testing was not carried out during the prelicense process then this testing should be conducted before the next batch of product is tested for mycoplasma contamination.

2.3.5 Test method

2.3.5.1 (**TO BE DETERMINED 3**) Inoculate each plate of each solid medium with 0.2 ml of product to be examined and inoculate 10 ml per at least 100 ml of each liquid medium. Incubate the agar plates at 35^0C to 38^0C, microaerophilically, for 10-14 days in an atmosphere of adequate humidity to prevent desiccation of the surface. Incubate the liquid media at 35^0 C to 38^0 C in (**TO BE DETERMINED 4**) air or microaerophilic conditions for 21 days. At the same time incubate an uninoculated 100 ml portion of each liquid medium and agar plates as a negative control. If any significant pH change occurs upon the addition of the product to be examined (this should be determined at the time of prelicense), the liquid medium shall be restored to its original pH value by the addition of a solution of either sodium hydroxide or hydrochloric acid. On day 2 or 3 after inoculation, subculture each liquid culture by inoculating at least 1 plate of each solid medium with 0.2 ml and incubate them at 35^0C to 38^0C

microaerophilically for 10-14 days. Repeat the procedure on the 6th or 7th day, again on the 13th or 14th day and again on the 20th or 21st day of the test. Incubate those agar plates inoculated on day 20 or 21 for 7 days. Observe the liquid medium or media every 2 or 3 days and if a color change occurs, subculture immediately.

2.3.5.2 If the liquid medium or media shows bacterial or fungal contamination, repeat the test. If any of the plates inoculated before the 6th or 7th day are broken or contaminated then the test must be repeated. As long as one of the plates inoculated on the 7th or 14th day can be read for mycoplasma contamination the test is considered valid and the bacteria or fungi contamination is ignored. Read all plates that can be read.

2.3.5.3 Include in the test, positive controls prepared by inoculating approximately 100 CFU (70-130 CFU) of one of the working reference species onto the agar plates and into the broth medium or media. This control shall be used on each test session conducted with a medium that has been validated for nutritive properties using working references determined by the types of products being tested as specified in **Section 2.3.1** of this guideline.

2.3.6 Judgement of the culture method

At the end of the incubation period, examine all the inoculated solid media microscopically for the presence of mycoplasma colonies. The product is negative for Mycoplasma contamination if the growth of Mycoplasma colonies has not occurred on any of the inoculated solid media. (**TO BE DETERMINED 9**) If growth of Mycoplasma colonies has occurred on any of the solid media, the test and sample tested are considered positive for Mycoplasma contamination. The test is invalid if the positive controls do not show growth of the relevant working reference organisms or the negative controls are positive for Mycoplasma contamination. If either of the controls is invalid the test must be repeated.

2.4 Indicator cell culture method

Cell cultures are stained with a fluorescent dye that binds to DNA. Mycoplasmas are detected by their characteristic particulate or filamentous pattern of fluorescence on the cell surface, and if contamination is heavy, in the surrounding areas.

2.4.1 Validation of the indicator cell culture method

Using a VERO or other equivalent in efficiency indicator cell culture substrate, validate the procedure using an inoculum of approximately 100 CFU (70-130 CFU) of working references of *M. hyorhinis* and *M. orale***.** Both working references must be positive when stained with the DNA stain at the end of the test.

If for viral, etc., suspensions the interpretation of results is affected by cytopathic effects, the virus may be neutralized using a specific antiserum that has no

inhibitory effects on mycoplasmas, or an alternative cell culture substrate that does not allow the growth of the virus may be used. To demonstrate the absence of inhibitory effects of serum, carry out the positive control tests in the presence of neutralizing antiserum.

2.4.2 Test method

2.4.2.1 Seed the indicator cell culture at a suitable density that will yield confluence of the cells after 3 days of growth (example: 2 X 10^4 to 2 X 10^5 cells per ml, 4 X 10^3 to 2.5 X 10^4 cells/cm^2) in a cell culture vessel of not less than 25 cm. Inoculate 1 ml of the sample to be examined into the cell culture vessel and incubate at 35^0 C to 38^0 C for at least 3 days.

2.4.2.2 After at least 3 days of incubation and the cells have grown to confluence, (**TO BE DETERMINED 6**) subculture 1 ml onto cover slips in suitable containers or on some other surface (chambered slides) suitable for the test procedure. Seed the cells in the second subculture at a low density so that they reach only 50 % confluence after 3-5 days of incubation. Complete confluence will impair visualization of mycoplasmas after staining.

2.4.2.3 Remove and discard medium from cover slips or chambered slides. Rinse the monolayer of indicator cells with Phosphate buffered saline (PBS) and then fix with glacial acetic acid/methanol (1 to 3) or some other suitable fixing solution.

2.4.2.4 Remove the fixing solution and discard. Wash off the fixing solution with sterile water and dry slides completely if they are to be stained later.

2.4.2.5 Add a suitable fluorescent dye that binds to DNA such as bisbenzamide stain (Hoechst) and allow to stain for a suitable time.

2.4.2.6 Remove the stain and rinse the monolayer with water. Mount the cover slips if applicable and examine the slides by epifluorescence (for bisbenzamide stain use a 330 nm/380 nm excitation filter, LP 440 nm barrier filter) at 100-400 X magnification or greater.

2.4.2.7 Compare the microscopic appearance of the test cultures with that of the negative and working reference controls, examining for extra nuclear fluorescence. Mycoplasmas produce pinpoints or filaments over the indicator cell's cytoplasm. They may also produce pinpoints and filaments in the intercellular spaces.

2.4.3 Judgement of the indicator cell culture method

The product being examined is negative for Mycoplasma contamination if there is no evidence of pinpoints or filaments of extranuclear fluorescence. If the slides inoculated with the product contain evidence of pinpoints or extranuclear fluorescence indicative of Mycoplasma the test and sample tested are considered positive for Mycoplasma contamination. The test is invalid if the positive controls do not show the presence of the appropriate extranuclear fluorescence of the reference organisms or the negative cell controls contain extranuclear fluorescence. If either of the controls is invalid the test must be repeated.

3. APPENDICES

3.1 Suggested broth and agar formulations

9 CFR Mycoplasma Broth

Heart Infusion Broth	62.5 gm
Proteose Peptone #3	25.0 gm
Yeast Extract	12.5 ml
1 % Thallium Acetate	62.5 ml
1 % Tetrazolium Chloride	13.75 ml
Penicillin (100,000 units/cc)	12.5 ml
Heat inactivated Horse Serum	250 ml
H_2O	2425 ml

Mix all ingredients well and then pH to 7.9 with 10 Normal NaOH.
Filter sterilize through a 0.2 um filter. Dispense into sterile test vessels.
Add DPN-Cysteine solution before use, 2 ml/100 ml of broth.

9 CFR Mycoplasma Agar

Heart Infusion Agar	25 gm
Heart Infusion Broth	10 gm
Proteose Peptone #3	10 gm
1% Thallium Acetate	25 ml
H_2O	995 ml
Heat Inactivated Horse Serum	126 ml
Yeast Extract	5 ml
Penicillin (100,000 units/cc)	5.2 ml
DPN-Cysteine	21 ml

Combine heart infusion agar, heart infusion broth, proteose peptone #3, Thallium acetate, and H_2O.
Mix and bring to boil, then cool. Adjust the pH to 7.9 with 10 Normal NaOH.
Autoclave 20 min. at 121^0 C. Cool in water bath to 56^0 C.
Aseptically add: horse serum, yeast extract, Penicillin, and DPN-Cysteine.
Dispense 12 ml into each 15 X 60 mm petri dish.

Japanese Liquid Medium for Mycoplasma

Basal Medium

50 % w/v Bovine Cardiac Muscle Extract	100 ml
Meat Peptone	10 gm
Sodium Chloride	5 gm
Glucose	1 gm
Sodium L-glutamate	0.1 gm
L-arginine hydrochloride	1 gm
H_2O	QS to 1000 ml

Filter sterilize through 0.22 um membrane filter or sterilize at 121^0 C for 15 min. Adjust the pH of the medium after sterilization to 7.2-7.4.

Additives for 77 ml of the Basal medium;

Horse Serum	10 ml
Inactivated Porcine Serum	5 ml
25 % w/v Fresh Yeast Extract	5 ml
1 % w/v B-NAD (oxidized)	1 ml
1 % w/v L-cysteine HCL (1 H_2O)	1 ml
0.2 % w/v phenol red	1 ml

Previously filter sterilize the additives and aseptically add to the sterilized basal medium. The additives which can be sterilized by high pressure can be autoclaved. Penicillin G potassium, 500 units/ml of the medium, or Thallium acetate, 0.02 % w/v, can be added.

Japanese Agar Medium for Mycoplasma

Basal Medium (C.3 above)	78 ml
Agar	1 gm

Sterilize by autoclaving 121^0 C for 15 min.

Additives:

Horse Serum	10 ml
Inactivated Porcine Serum	5 ml
25 % w/v fresh yeast extract	5 ml
1 % w/v B-NAD (oxidized)	1 ml
1 % w/v L-cysteine HCL (1 H_2O)	1 ml

Penicillin G potassium, 500 units per ml of medium, or thallium acetate, 0.02 % w/v can be added.

Add the additives to basal/agar medium which has been liquefied by heating, and divide into sterile petri dishes, 45-55 mm. Cool and allow to solidify.

EP Recommended media for detection of *M. Gallisepticum*

Liquid Medium;

Beef Heart Infusion Broth (1)	90 ml
Horse Serum (unheated)	20 ml
Yeast Extract (250 gm/l)	10 ml
Thallium Acetate (10 gm/l solution)	1 ml
Phenol Red (0.6 gm/l solution)	5 ml
Penicillin (20,000 I.U. per milliliter)	0.25 ml
Deoxyribonucleic acid (2 gm/l solution)	1.2 ml

Adjust to pH 7.8.

Solid Medium;

Prepare as described for the liquid medium above but replace beef heart infusion broth with beef heart infusion agar containing 15 gm/l of agar.

EP Recommended Media for the detection of *M. Synoviae*

Liquid Medium:

Beef Heart Infusion Broth (1)	90 ml
Essential Vitamins (2)	0.025 ml
Glucose monohydrate (500 gm/l solution)	2 ml
Swine serum (inactivated at 56^0 C for 30 min.)	12 ml
B-Nicotinamide adenine dinucleotide (10 gm/l solution)	1 ml
Cysteine hydrochloride (10 gm/l solution)	1 ml
Phenol Red (0.6 gm/l solution)	5 ml
Penicillin (20,000 I.U. per milliliter)	0.25 ml

Mix the solutions of B-nicotinamide adenine dinucleotide and cysteine hydrochloride and after 10 min. Add the other ingredients. Adjust to pH 7.8

Solid Medium:

Beef Heart Infusion Broth (1)	90 ml
Ionagar (3)	1.4 gm

Adjust pH to 7.8, and sterilize by autoclaving, then add:

Essential Vitamins (2)	0.025 ml
Glucose monohydrate (500 gm/l solution)	2 ml
Swine serum (unheated)	12 ml
B-Nicotinamide adenine dinucleotide (10gm/l solution)	1 ml
Cysteine hydrochloride (10 gm/l solutions)	1 ml
Phenol Red (0.6 gm/l solution)	5 ml
Penicillin (20,000 I.U. per milliliter)	0.25 ml

EP Recommended Media for the Detection of Non-avian Mycoplasma

Liquid Medium:

Hank's Balanced Salt Solution (modified) (4)	800 ml
H_2O	67 ml
Brain Heart Infusion (5)	135 ml
PPLO Broth	248 ml
Yeast Extract (170 gm/l)	60 ml
Bacitracin	250 mg
Meticillin	250 mg
Phenol Red (5 gm/L)	4.5 ml
Thallium Acetate (56 gm/l)	3 ml
Horse Serum	165 ml
Swine Serum	165 ml

Adjust the pH to7.4-7.45

Solid Medium:

Hank's Balance Salt Solution (modified) (4)	200 ml
DEAE-dextran	200 ml
Ionagar (3)	15.65 gm

Mix well and sterilize by autoclaving. Cool to 100^0 C. Add this to 1740 ml of the liquid medium described above.

EP Media Sub parts

(1) Beef Heart Infusion Broth

Beef Heart (for preparation of the infusion)	500 gm
Peptone	10 gm
Sodium Chloride	5 gm
H_2O	QS to 1000 ml

Sterilize by autoclaving.

(2) Essential Vitamins

Biotin	100 mg
Calcium pantothenate	100 mg
Choline chloride	100 mg
Folic acid	100 mg
i-Inositol	200 mg
Nicotinamide	100 mg
Pyridoxal hydrochloride	100 mg
Riboflavin	10 mg
Thiamine hydrochloride	100 mg
H_2O	QS to1000 mg

(3) Ionagar

A highly refined agar for use in microbiology and immunology, prepared by an ion-exchange procedure which results in a product having superior purity, clarity, and gel strength.

It contains approximately:

H_2O	12.2 per cent
Ash	1.5 per cent
Acid insoluble ash	0.2 per cent
Chlorine	0.0
Phosphate (calculated as P_2O_5)	0.3 per cent
Total Nitrogen	0.3 per cent
Copper	8 ppm
Iron	170 ppm
Calcium	0.28 per cent
Magnesium	0.32 per cent

(4) Hank's balanced salt solution (modified)

Sodium chloride	6.4 gm
Potassium chloride	0.32 gm
Magnesium sulphate heptahydrate	0.08 gm
Magnesium chloride hexahydrate	0.08 gm
Calcium chloride, anhydrous	0.112 gm
Disodium hydrogen phosphate dihydrate	0.0596 gm
Potassium dihydrogen phosphate, anhydrous	0.048 gm
H_2O	QS to 800 ml

(5) Brain heart infusion

Calf brain infusion	200 gm
Beef heart infusion	250 gm
Proteose peptone	10 gm
Glucose	2 gm
Sodium chloride	5 gm
Disodium hydrogen phosphate, anhydrous	2.5 gm
H_2O	QS to 1000 ml

(6) PPLO broth

Beef heart infusion	50 gm
Peptone	10 gm
Sodium chloride	5 gm
H_2O	QS to 1000 ml

3.2 REFERENCE PRODUCTION AND UTILIZATION

(**TO BE DETERMINED 2**) The Master references of the 5 strains of Mycoplasma listed in **Section 2.3.1** will be isolated by labs of the European Union and donated to the European Department of the Quality of Medicines (EDQM). EDQM will produce a sufficient quantity of these Master references to be distributed to the 3 regional government laboratories (Japan, EU, and USA) of this VICH Mycoplasma working group. The regional government labs will then distribute these master references to those labs in their region wishing to be validated in Mycoplasma testing. The producing European labs will make the references so that they contain approximately 100 CFU (70-130 CFU) per specified inoculum. A group of laboratories in the three regions will standardize these references and validate the CFUs.

For Mycoplasma test validation each laboratory will be sent 3 vials of each reference strain depending on the types of products being tested. A different production lot of the media or medias shall be used for each vial of a reference strain. After the laboratory completes the testing they shall report their results to the regional government laboratory supplying the reference vials. At the time of the validation testing each laboratory shall produce and validate working references from the Master references. These working references shall be used to test subsequent batches of Mycoplasma media used in the labs testing for Mycoplasma contamination in veterinary biologics.